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Alteration of a Cyclic AMP-Dependent Protein Kinase Phosphorylation Site in the c-Fos Protein Augments Its Transforming Potential

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We have studied the phosphorylation of the nuclear oncoprotein Fos by cyclic AMP-dependent protein kinase (PKA). We demonstrate that the human c-Fos protein, phosphorylated either in vitro with purified PKA or in vivo in JEG3 cells following treatment with forskolin, has similar phosphotryptic peptide maps. Serine 362, which constitutes part of a canonical PKA phosphorylation site (RKGSSS), is phosphorylated both in vivo and in vitro. A mutant of Fos protein in which serine residues 362 to 364 have been altered to alanine residues is not efficiently phosphorylated in vitro. Furthermore, Fos protein in which serines 362 to 364 have been altered to alanine shows increased transforming potential. We propose that phosphorylation of Fos by PKA is an important regulatory step in controlling its activity in normal cell growth and differentiation.

The product of the proto-oncogene *fos* is a nuclear phosphoprotein (12). It belongs to the family of transcription cofactors which, upon association with members of the Jun family, can activate transcription from promoters containing an AP-1 site (10, 34). Although the calculated molecular mass of the Fos protein is about 42 kDa (380 amino acids), it migrates on a sodium dodecyl sulfate (SDS)-polyacrylamide gel as a heterogeneous protein with an approximate molecular mass of 55 to 65 kDa (3, 12). This heterogeneity is largely due to phosphorylation of serine residues (3). Although both c-Fos and its viral homolog v-Fos are phosphorylated, the cellular protein is more extensively phosphorylated (3). Major phosphorylation sites in c-Fos have been localized to its C terminus within the amino acid sequence that differs from that of v-Fos (3, 41). Precise identification of the serine residues phosphorylated, as well as the kinases involved in this phosphorylation, would help our understanding of the biological function of Fos phosphorylation.

A large body of data implicates the involvement of the cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway in the regulation of both the *fos* gene and the Fos protein (31). Activation of the PKA pathway by adenyl cyclase agonists such as forskolin leads to Fos expression (2, 7, 8, 21, 26), presumably via interaction of the phosphorylated cAMP responsive element (CRE)-binding protein with the CRE in the *fos* promoter (25, 36). Additionally, it has been shown that the subcellular localization and the DNA-binding activity of the Fos protein may be regulated by interaction with proteins whose activities are under the control of the PKA pathway (2, 32).

We have investigated whether PKA might also act at the posttranslational level by directly phosphorylating the Fos protein. Indeed, consensus sequences for phosphorylation by PKA can be identified in the Fos protein. We report that PKA does phosphorylate the human c-Fos protein in vitro on a site also phosphorylated in vivo. We have identified serine residue 362 as the phosphorylated amino acid. Mutation of serine residues 362 to 364 to alanine enhances the transforming activity of c-Fos to levels comparable with those of v-Fos. It has previously been shown that lack of phosphorylation of c-Fos at its C terminus abolishes its ability to downregulate its own promoter but has no effect on transactivation of genes linked to an AP-1 site (29, 36). Our results suggest that part of the mechanism that leads to cell transformation by Fos might be attributed to the loss of a PKA phosphorylation site in v-Fos. Moreover, our results implicate PKA in regulation of Fos activity in a complex manner by controlling both induction and repression of the *fos* promoter.

MATERIALS AND METHODS

Cell lines and culture conditions. The human choriocarcinoma cell line JEG3 (20) was maintained in Dulbecco-Vogt modified Eagle medium (DMEM) containing 5% fetal bovine serum and 5% equine serum. Cos7 cells were maintained in DMEM containing 10% bovine calf serum.

Plasmids and cell transfection. For expression in Cos7 cells, the wild-type and mutant Fos cDNAs were cloned in pSG424 (33) from which *GAL4* coding sequences were excised by digestion with *Bgl*II and *Bam*HI and religated. SGFos (wild type [WT] Fos), SGFosSerA (FosSerA), SG FosSerB (FosSerB), and SGFosSerC (FosSerC) were generated by cloning a *Kpn*I-*Eco*RI cDNA fragment from pBK28 (35) and FosSerA, FosSerB, and FosSerC into pSG424. The construction of mutants FosSerA, FosSerB, and FosSerC has been described elsewhere (29).

Transfections were performed by the DEAE-dextran method (24). Briefly, 5×10^5 cells were seeded in 35-mm tissue culture dishes. The medium was changed to DMEM containing 10% fetal calf serum (FCS) and 100 mg of chloroquine per ml. One microgram of DNA was added in 200 μl of the same medium containing 100 μg of DEAE-dextran per ml. After 4 to 5 h at 37°C, cells were incubated for 5 min at 37°C in 10% dimethyl sulfoxide in phosphate-

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buffered saline. The cells were washed once and incubated for 48 h in DMEM containing 10% FCS before metabolic labeling or preparation of unlabeled cell extracts.

Focus assay. 208F cells were seeded 24 h before transfection at a density of 5×10^5 cells per 10-cm tissue culture dish and maintained in DMEM containing 10% FCS. Cells were transfected with 6 μg of plasmid DNA by the calcium phosphate precipitation method (9) and exposed to the precipitate for 12 to 16 h. After transfection, cells were split 1:3 and fed with DMEM supplemented with 10% FCS and 10^6 M dexamethasone (Sigma). Two to three weeks post-transfection, foci of piled-up round cells were counted. The plasmids used were pBK28 (encoding human c-Fos), Fos SerA (encoding the human c-Fos serine mutant A), and FBR-MuSV (v-Fos).

Metabolic labeling and immunoprecipitation. For labeling with [^{35}S]methionine, JEG3 and Cos7 cells were incubated for 30 min at 37°C in methionine-free medium and then for 2 h at 37°C in methionine-free DMEM containing 10% dialyzed FCS and 200 μCi of Translabel (ICN Pharmaceutical Co.) per ml. Unless otherwise specified, JEG3 cells were then treated for 90 min with 50 μM forskolin (Sigma) before proteins were extracted.

For ^{32}P labeling, JEG3 cells and Cos7 cells were incubated for 1 h at 37°C in phosphate-free DMEM containing 10% dialyzed FBS and then for 3 (Cos7 cells) or 6 (JEG3 cells) h in phosphate-free DMEM containing 10% dialyzed FBS and 2 mCi of $^{32}P_i$ (Dupont, NEN Research Products) per ml. JEG3 cells were subsequently treated for 90 min with 50 μM forskolin before harvesting.

After labeling, cells were washed with Tris-buffered saline and solubilized in 1 ml of lysis buffer (10 mM Tris hydrochloride [pH 7.5], 0.05 M NaCl, 1% deoxycholate, 1% Nonidet P-40, 1 mM aprotinin [Sigma], 1 mM phenylmethylsulfonyl fluoride [Sigma]) per 10-cm tissue culture dish. Lysates were precleared by incubation with 50 μl of Pansorbin (Calbiochem) for 30 min on ice and centrifugation for 60 min at 20,000 × *g* at 4°C to remove insoluble debris. Supernatants were incubated for 1 h with anti-Fos monoclonal antibody 18H6 (14) (about 1 μl/ml of cell lysate) and then with 50 μl of Pansorbin for 60 min on ice. The immunoprecipitates were collected by centrifugation for 1 min in a microcentrifuge at 4°C and were washed three times with RIPA buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 1% aprotinin). The precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (22). When proteins were labeled with [^{35}S]methionine, the gel was incubated for 30 min in En^{3}Hance (Dupont, NEN Research Products) prior to drying.

For use in in vitro kinase assay, Fos immunoprecipitates were obtained from transfected Cos7 cells as described above except that protein A-Sepharose (Repligen) instead of Pansorbin was used to precipitate the immune complexes.

In vitro kinase assay. In vitro phosphorylation with the purified catalytic subunit of PKA purified from rat liver and kindly provided by Ushio Kikkawa was performed with human c-Fos protein purified from Sf9 cells infected with a Fos recombinant baculovirus (AcFos) (40) or with Fos protein expressed and immunoprecipitated from transfected Cos7 cells as described above. Immune complexes were washed three times with kinase buffer (20 mM Tris hydrochloride [pH 7.4], 10 mM $MgCl_2$, 1 mM dithiothreitol) and then incubated for 10 or 30 min at 30°C in 30 μl of kinase buffer in the presence of 0.05 μg of PKA catalytic subunit and 1 μl of [γ-^{32}P]ATP (3,000 μCi/mmol; Amersham). Purified AcFos was incubated in similar conditions, using about 1 μg of purified protein per reaction. After incubation, the reaction mixture was analyzed by SDS-PAGE. The gel was rinsed in water, dried, and exposed to XAR film.

Two-dimensional tryptic peptide analysis. Gel-purified ^{32}P-labeled Fos protein was oxidized and digested with trypsin (Worthington) as described previously (6). Samples were spotted and separated onto cellulose thin-layer chromatography (TLC) plates (20 by 20 cm; EM Science) and subjected to electrophoresis at pH 1.9 (50 parts 88% formic acid, 156 parts glacial acetic acid, 1,796 parts deionized water) for 40 min at 1.0 kV in the first dimension and chromatography in phosphochromo buffer (750 parts *n*-butanol, 500 parts pyridine, 150 parts glacial acetic acid, 600 parts deionized water) for 10 h in the second dimension. Plates were air dried and exposed to XAR film at −70°C with an intensifying screen.

Partial acid hydrolysis and manual Edman degradation. Individual ^{32}P-labeled peptides were isolated from TLC plates as described previously (6). For partial acid hydrolysis, the eluted peptides were incubated for 30 min at 110°C in 6 N HCl, lyophilized, dissolved in 6 μl of deionized water, and spotted on TLC plates. The hydrolysis products were resolved by electrophoresis at pH 3.5 (100 parts glacial acetic acid, 10 parts pyridine, 1,890 parts deionized water) for 30 min at 1.5 kV in the first dimension and by chromatography for 10 h in phosphochromo buffer in the second dimension.

For manual Edman degradation, the eluted peptide was treated as described previously (6). Briefly, 1 volume of 5% phenylisothiocyanate in pyridine was added to 1 volume of the peptide dissolved in deionized water (20 μl), and the mixture was incubated for 30 min at 45°C. The sample was then extracted twice with 200 μl of heptane-ethyl acetate (10:1) and twice with 200 μl of heptane-ethyl acetate (2:1). The aqueous phase was lyophilized; the dried pellet taken up in 50 μl of trifluoroacetic acid and incubated for 10 min at 45°C. The trifluoroacetic acid was lyophilized, and the sample was resuspended in deionized water. An aliquot was removed, and the rest of the reaction mixture subjected to a new round of degradation. The reaction products were analyzed by electrophoresis for 20 min at 1.5 kV in pH 1.9 buffer on a TLC plate.

RESULTS

Induction and phosphorylation of Fos protein in JEG3 cells by forskolin. Forskolin is an agonist of adenylate cyclase, which, when added to cells, leads to activation of the PKA pathway (38). It has previously been shown that activation of the PKA pathway results in the induction of Fos expression in various cell lines (2, 7, 8, 21, 26). When JEG3 cells, a human choriocarcinoma cell line responsive to forskolin (13), were treated with forskolin, synthesis of Fos protein could be observed. Figure 1A shows the Fos protein immunoprecipitated from ^{35}S-labeled JEG3 cells at different times after treatment with forskolin. The lower-molecular-weight form of Fos protein detected after 30 min of forskolin addition is progressively converted into a higher-molecular-weight form (Fig. 1A, lanes 2 to 6). The modified forms of the Fos protein are likely to result from phosphorylation, since treatment of the immunoprecipitates with bacterial alkaline phosphatase converts the slowly migrating species to the faster-moving form (Fig. 1B; compare lanes 7 and 8).

Fos protein is a substrate for PKA in vitro. Since forskolin treatment results in an increase of intracellular cAMP levels and activation of the PKA pathway, we investigated whether

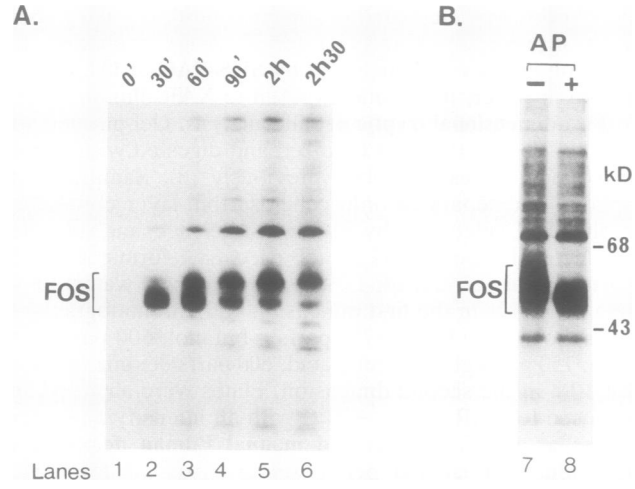


FIG. 1. Metabolic labeling of endogenous human c-Fos protein in JEG3 cells. JEG3 cells were treated with forskolin and labeled with [^{35}S]methionine for various times. Extracts were prepared and immunoprecipitated with anti-Fos antibody prior to separation by SDS-PAGE as described in Materials and Methods. (A) Time course of expression of Fos in JEG3 cells. (B) JEG3 cells were treated with forskolin and labeled with ^{35}S-amino acids for 1 h. Half of the immunoprecipitate was treated with bacterial alkaline phosphatase (AP) for 1 h at 37°C as described previously (3).

Fos might be a substrate for PKA. We have previously expressed the human Fos protein in insect cells by using a recombinant baculovirus (40). We therefore used the Fos protein purified from this source (AcFos) as a substrate for the catalytic subunit of PKA. As shown in Fig. 2A, AcFos was labeled in vitro after incubation with PKA and [γ-^{32}P]ATP (lanes 1 and 2). When either AcFos or PKA was omitted, the 55- to 65-kDa phosphoprotein was not detected (lanes 3 and 4, respectively), attesting to the specificity of the reaction. Other bands detected together with Fos in lanes 1 and 2 are contaminants in the AcFos or PKA preparation or represent degradation products of the purified protein.

To determine the biological relevance of Fos phosphorylation by PKA, we investigated whether it occurred at sites normally phosphorylated in vivo. To do so, we compared the tryptic phosphopeptide maps of Fos protein immunoprecipitated from JEG3 cells metabolically labeled with $^{32}P_i$ following treatment with forskolin and of AcFos phosphorylated in vitro by PKA.

Figure 2B shows the phosphotryptic peptide maps of the in vivo- and in vitro-labeled Fos proteins. Four peptides, labeled 1 to 4, were identified in the in vivo-labeled Fos protein, whereas three peptides with mobilities similar to those of peptides 1, 3, and 4 (marked as 1′, 3′, and 4′) could be identified in the in vitro-labeled AcFos protein. When the tryptic digests of the in vivo- and in vitro-labeled proteins were mixed, no new additional peptides could be detected. Thus, it appears that peptide 2 observed in the in vivo-labeled Fos protein represents either a unique phosphorylated site or a partially digested tryptic peptide. Since the in vitro- and in vivo-labeled proteins had rather similar peptide maps, it appears likely that Fos is a substrate of PKA in vivo.

Fos is phosphorylated by PKA at its C terminus. We have previously shown that several serine residues, located within the C-terminal 40 amino acids of the Fos protein, are phosphorylated upon treatment of NIH 3T3 cells with tetradecanoyl phorbol acetate (3). We have shown here that Fos can be a substrate for PKA, and cross-talk between PKC and PKA pathways is not unprecedented (30, 35). Moreover, serine residue 362 of Fos protein (Fig. 3A) is located within an excellent consensus sequence for PKA (19). This finding prompted us to investigate whether the sites that are phosphorylated by PKA might be located

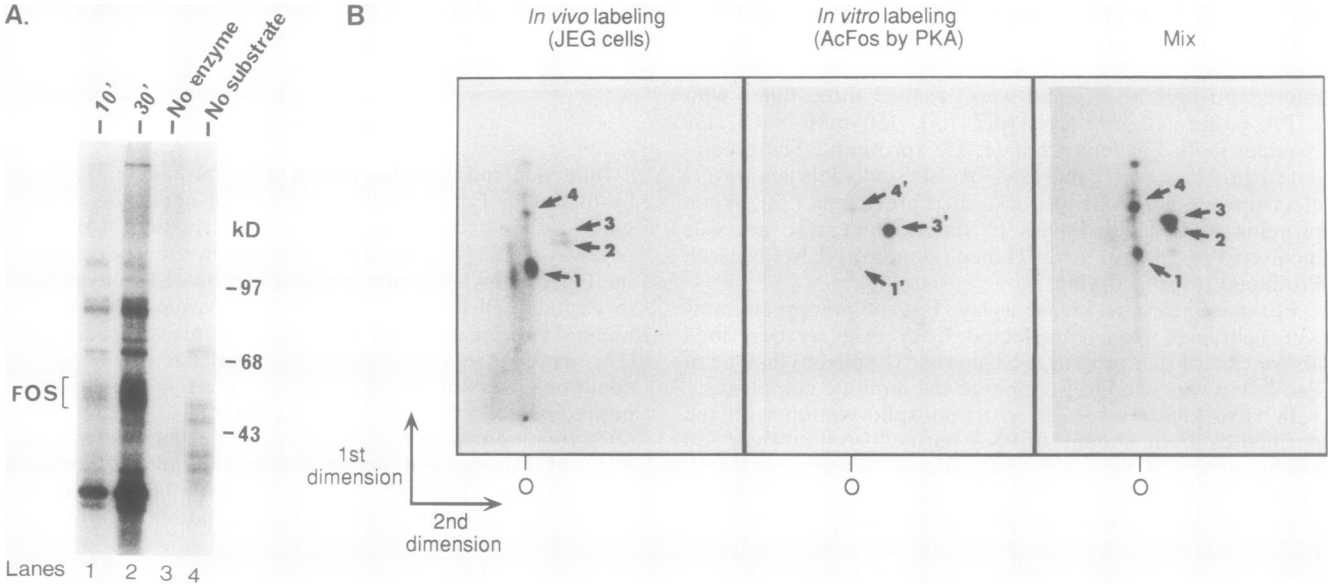
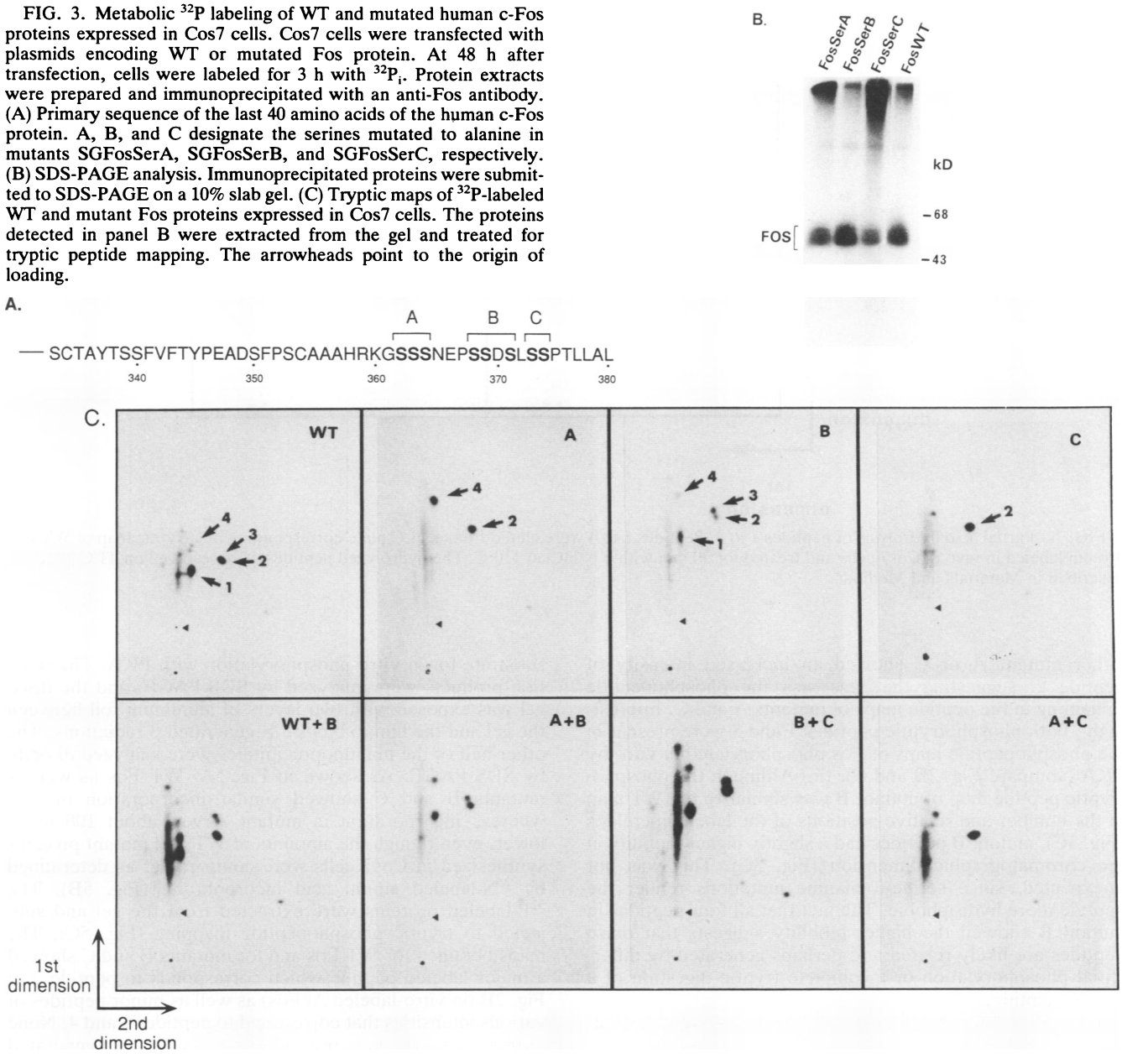


FIG. 2. In vitro phosphorylation of human c-Fos protein by PKA. (A) Human c-Fos protein purified from insect cells infected with a Fos recombinant baculovirus (AcFos) was incubated with purified PKA at 30°C in the presence of [γ-^{32}P]ATP. Lanes: 1, 10-min incubation; 2, 30-min incubation; 3, PKA was omitted; 4, Fos protein was omitted. (B) Tryptic peptide maps of ^{32}P-labeled Fos protein. Fos protein was immunoprecipitated either from metabolically labeled JEG3 cells or after in vitro labeling by PKA as for panel A. The labeled proteins were extracted from gel and treated for tryptic peptide mapping as described in Materials and Methods.

FIG. 3. Metabolic ^{32}P labeling of WT and mutated human c-Fos proteins expressed in Cos7 cells. Cos7 cells were transfected with plasmids encoding WT or mutated Fos protein. At 48 h after transfection, cells were labeled for 3 h with $^{32}P_i$. Protein extracts were prepared and immunoprecipitated with an anti-Fos antibody. (A) Primary sequence of the last 40 amino acids of the human c-Fos protein. A, B, and C designate the serines mutated to alanine in mutants SGFosSerA, SGFosSerB, and SGFosSerC, respectively. (B) SDS-PAGE analysis. Immunoprecipitated proteins were submitted to SDS-PAGE on a 10% slab gel. (C) Tryptic maps of ^{32}P-labeled WT and mutant Fos proteins expressed in Cos7 cells. The proteins detected in panel B were extracted from the gel and treated for tryptic peptide mapping. The arrowheads point to the origin of loading.



within Fos C-terminal sequences. We therefore generated mutants of Fos protein in which the serine residues were altered to alanine (Fig. 3A) (29). In all cases, a group of serine residues, serines 362, 363, and 364 for mutant A, serines 368, 369, and 371 for mutant B, and serines 373 and 374 for mutant C, were altered to alanine. WT and C-terminal-mutated Fos cDNAs (FosSerA, FosSerB, and FosSerC) were transiently expressed at high levels in Cos7 cells. Neither tetradecanoyl phorbol acetate nor forskolin treatment resulted in the induction of expression of the endogenous *fos* gene in Cos7 cells (data not shown), thus allowing us to express mutated proteins without interference of the endogenous WT Fos protein.

The mutant Fos proteins expressed in Cos7 cells were labeled efficiently in vivo in the presence of $^{32}P_i$. As shown in Fig. 3B, all three mutants incorporated $^{32}P_i$. No obvious difference between the apparent molecular weights of the labeled WT and mutant proteins was detected. In contrast, mutant A transfected in NIH 3T3 cells was very poorly phosphorylated (29). Perhaps overproduction of Fos in Cos7 cells masks the differences in the extent of phosphorylation by the three mutants. Alternatively, other protein kinases in Cos7 cells can phosphorylate the serine residues at B and C sites. The labeled Fos proteins were extracted from the gel and subjected to phosphotryptic peptide analysis. The results shown in Fig. 3C indicate the following. (i) The phosphotryptic peptide maps of mutants A and C were different from that of mutant B, which was nearly identical to that of the WT protein. Thus, mutation in serine residues at positions 362 to 364 (mutant A) and 373 to 374 (mutant C) affected phosphorylation of the Fos protein. (ii) Comparison of phosphotryptic peptides of the WT and mutant proteins show that peptides 1 and 3 are not present in mutants A and C (Fig. 3C). Furthermore, mixed maps of mutant B with

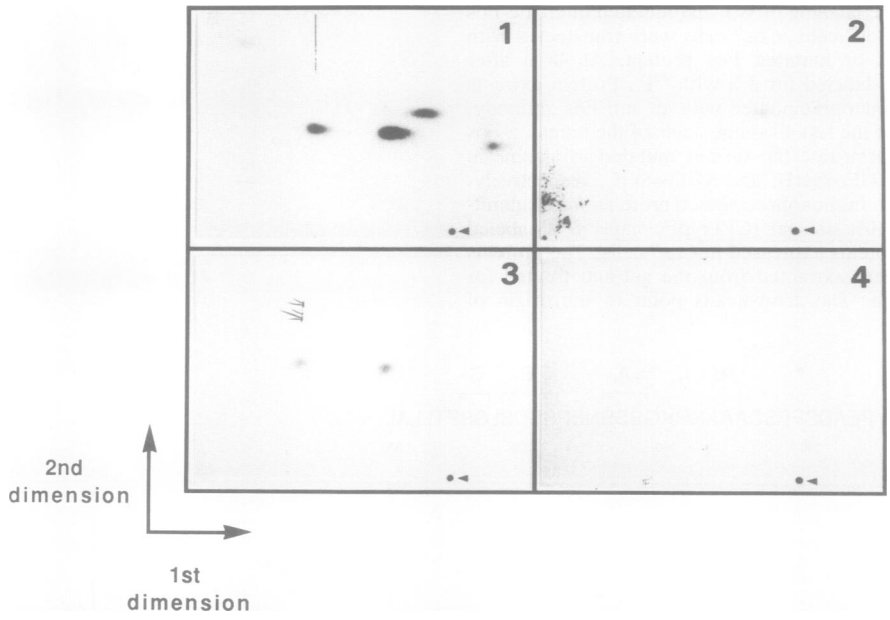


FIG. 4. Partial acid hydrolysis of peptides 1 to 4. Peptides 1 to 4 were eluted from a TLC plate corresponding to a tryptic map of WT Fos protein labeled in vivo in Cos7 cells and treated for 30 min with 6 N HCl at 110°C. The hydrolyzed peptides were resolved on TLC plates as described in Materials and Methods.

either mutant A or C showed an increased intensity of peptide 2, suggesting that this was the phosphopeptide remaining in the peptide maps of mutants A and C. Interestingly, both phosphotryptic peptides 1 and 3 were present in the phosphopeptide maps of Fos phosphorylated in vitro by PKA (compare Fig. 2B and C). (iii) Although the phosphotryptic peptide map of mutant B was similar to the WT map in the number and relative positions of the labeled peptides (Fig. 3C), mutant B peptides had a slightly higher mobility in the chromatographic dimension (Fig. 3C). This was not unexpected, since serine-to-alanine mutations render the peptide more hydrophobic. The fact that all four peptides in mutant B showed the higher mobility suggests that these peptides are likely related and perhaps generated by differential phosphorylation or incomplete tryptic digestion of a unique peptide.

To confirm that the labeled peptides 1 to 4 were related, the corresponding spots were extracted from the TLC plate corresponding to the tryptic map of WT Fos and subjected to partial acid hydrolysis as described in Materials and Methods. A given peptide will have a diagnostic pattern upon acid hydrolysis dictated by its primary structure. Therefore, related peptides should show common spots in their patterns of partial acid hydrolysis. As shown in Fig. 4, the hydrolysis patterns obtained for peptides 1 to 4 show at least some common spots, indicating that peptides 1 to 4 result from different modification of the same peptide.

In vitro phosphorylation of Fos mutants. Only the group of serine residues mutated in FosSerA, not those mutated in FosSerC, are contained within a canonical consensus sequence for PKA phosphorylation (19). Therefore, one would expect that only mutation of the group of serine A would prevent phosphorylation of Fos by PKA in vitro. To test this hypothesis, WT Fos and FosSerA, FosSerB, and FosSerC mutant DNAs were transfected into Cos7 cells and metabolically labeled with [^{35}S]methionine. Labeled Fos protein was immunoprecipitated with Fos antibody and used as the substrate for in vitro phosphorylation with PKA. The reaction products were analyzed by SDS-PAGE, and the dried gel was exposed with two layers of aluminum foil between the gel and the film to block ^{35}S-generated β radiations. The other half of the immunoprecipitates were analyzed directly by SDS-PAGE. As shown in Fig. 5A, WT Fos as well as mutants B and C showed similar incorporation of ^{32}P, whereas incorporation in mutant A was about 100 times lower, even though the amounts of WT and mutant proteins synthesized in Cos7 cells were comparable, as determined by ^{35}S-labeled amino acid incorporation (Fig. 5B). The ^{32}P-labeled proteins were extracted from the gel and subjected to tryptic phosphopeptide mapping (Fig. 5C). The maps obtained for WT Fos and for mutants B and C showed a major labeled peptide which corresponds to peptide 3 in Fig. 2B (in vitro-labeled AcFos) as well as minor peptides of various intensities that correspond to peptides 1 and 4. None of these peptides were present on the tryptic map generated from mutant A. This result strongly suggests that one or more of the serine residues located at positions 362 to 364 are phosphorylated by PKA.

To confirm that the serine residues mutated in Fos mutant A were phosphorylated in the WT Fos protein, we eluted peptide 3 from the TLC plate of the wild-type tryptic map and subjected it to several rounds of manual Edman degradation. The C-terminal tryptic peptide of Fos protein should be generated by cleavage at lysine 360 (Fig. 3A). However, it is known that phosphorylation close to a trypsin cleavage site can inhibit digestion at this site (18). Therefore, if Fos is phosphorylated at residue 362, which is the first serine position expected to be phosphorylated by PKA within the sequence RKGSSS (amino acids 359 to 364) trypsin would cleave after arginine 359 instead of lysine 360. Manual Edman degradation, which releases N-terminal amino acids, would then be expected to release free phosphate after the third round of degradation if serine 362 is phosphorylated. As shown in Fig. 6, this is indeed the case. Before any

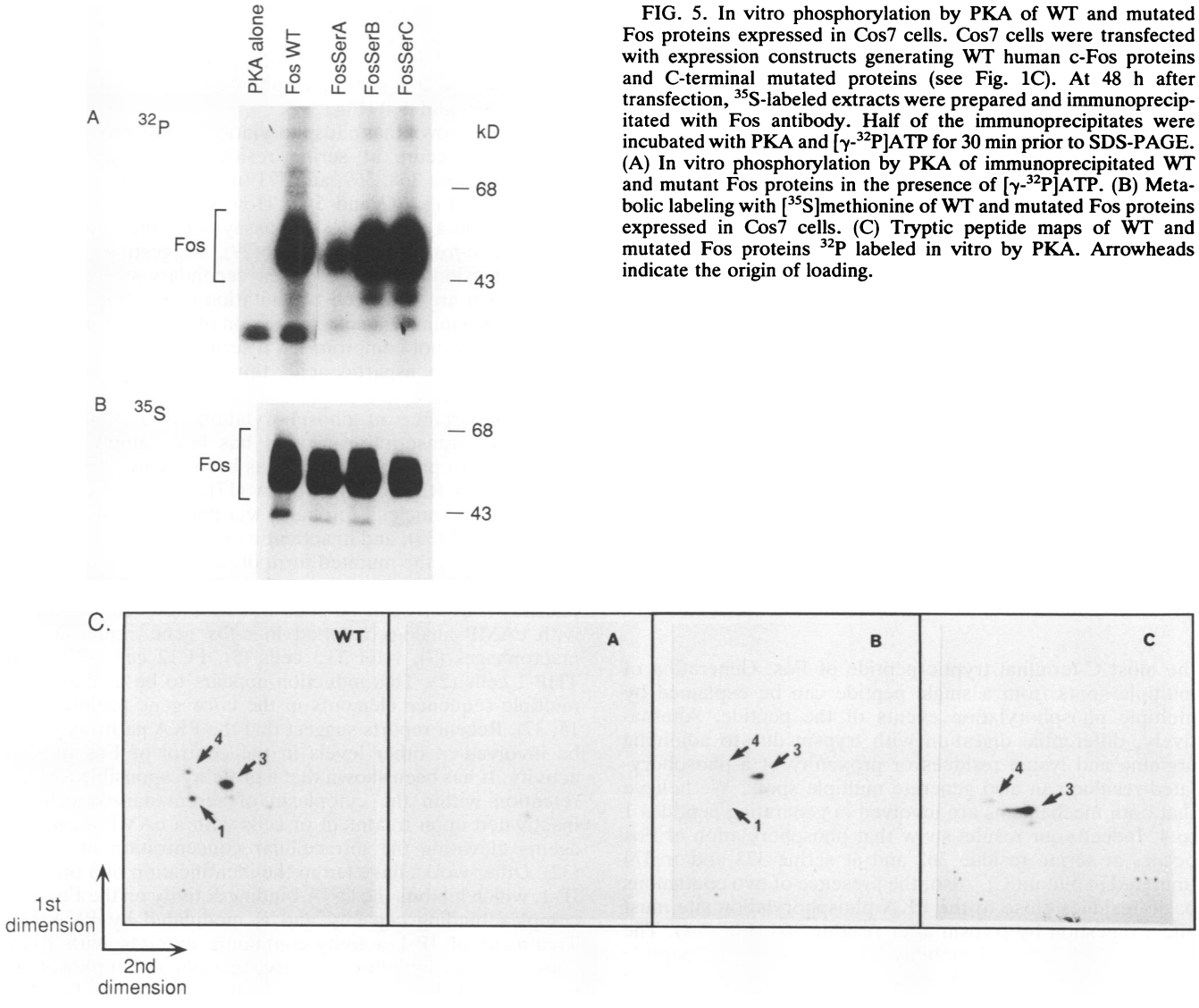


FIG. 5. In vitro phosphorylation by PKA of WT and mutated Fos proteins expressed in Cos7 cells. Cos7 cells were transfected with expression constructs generating WT human c-Fos proteins and C-terminal mutated proteins (see Fig. 1C). At 48 h after transfection, ^{35}S-labeled extracts were prepared and immunoprecipitated with Fos antibody. Half of the immunoprecipitates were incubated with PKA and [γ-^{32}P]ATP for 30 min prior to SDS-PAGE. (A) In vitro phosphorylation by PKA of immunoprecipitated WT and mutant Fos proteins in the presence of [γ-^{32}P]ATP. (B) Metabolic labeling with [^{35}S]methionine of WT and mutated Fos proteins expressed in Cos7 cells. (C) Tryptic peptide maps of WT and mutated Fos proteins ^{32}P labeled in vitro by PKA. Arrowheads indicate the origin of loading.

reaction was performed, peptide 3 migrated in the direction of the anode at pH 3.5 because of its overall positive charge. The first round of degradation resulted in a decrease in the positive charge of the peptide which migrated closer to the origin. The third round of degradation resulted in release of free phosphate, indicating that the third N-terminal amino acid of peptide 3 was phosphorylated. These results are consistent with peptide 3 being the most C-terminal tryptic peptide of Fos phosphorylated at serine residue 362.

Mutation of the PKA phosphorylation site increases c-Fos transforming potential. We have previously shown that serine residues 362 to 364 were phosphorylated and that this phosphorylation was required for Fos to manifest its transrepressing activity of its own promoter (29). Here, we have precisely identified serine residue 362 as a site of phosphorylation by PKA. To further delineate the biological importance of phosphorylation at this site of Fos protein, we tested the transforming potential of mutant FosSerA on 208F cells and compared it with those of WT Fos and FBR-MuSV (v-Fos). As shown in Table 1, mutation of serine residues 362 to 364 resulted in enhanced transforming capacity of FosSerA compared with c-Fos. The number of foci detected in transfected 208F cells was two to five times higher with FosSerA than with WT Fos. In fact, mutant FosSerA transformed with the same efficiency as did FBR-MuSV (v-Fos). Our results suggest that loss of a site of phosphorylation by PKA at its C terminus might be one of the crucial events that makes v-Fos a highly transforming protein compared with c-Fos.

DISCUSSION

We have demonstrated that human c-Fos protein is phosphorylated in vitro by PKA. This phosphorylation occurs on a site normally phosphorylated in vivo upon treatment of JEG3 cells with forskolin. We have identified the serine residue modified as serine 362. Earlier studies showed that mutation of serine 362 to alanine resulted in loss of transrepression activity of Fos on its own promoter (29). We show here that this mutation enhances c-Fos transforming potential to a level comparable to that of v-Fos.

The four major tryptic peptides detected on a tryptic map of WT Fos labeled in vivo with ^{32}P are related and therefore generated by a single tryptic peptide which corresponds to

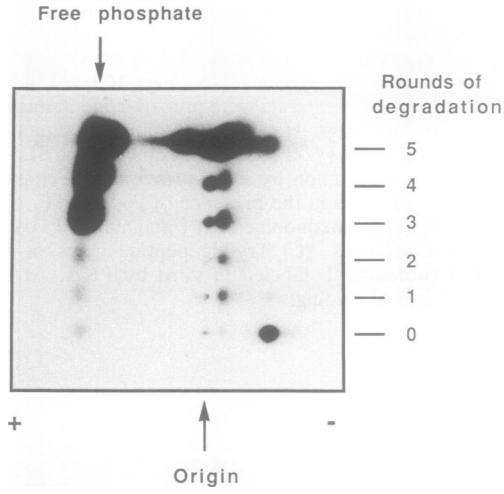


FIG. 6. Manual Edman degradation of peptide 3. WT Fos protein was phosphorylated in vitro by PKA and subjected to two-dimensional tryptic peptide analysis. Peptide 3 was eluted from the TLC plate. A total of 1,200 cpm was submitted to five rounds of degradation. Aliquots of 200 cpm were taken after every round of degradation and submitted to two-dimensional separation on a TLC plate as described in Materials and Methods.

the most C-terminal tryptic peptide of Fos. Generation of multiple spots from a single peptide can be explained by multiple phosphorylation events of the peptide. Alternatively, differential digestion with trypsin due to adjoining arginine and lysine residues or proximity of a phosphorylated residue can also generate multiple spots. We believe that both mechanisms are involved in generating peptides 1 to 4. Indeed, our results show that phosphorylation of Fos occurs at serine residue 362 and at serine 373 and/or 374 (mutated in mutant C). Also, the presence of two contiguous basic residues close to the PKA phosphorylation site must affect digestion by trypsin after residue 360 (Fig. 3A). The calculated charge and mobility of Fos C-terminal peptide phosphorylated once or twice fit with spot 3 being the result of a single phosphorylation event, whereas spot 1 would result from a double-phosphorylation event. Comparison of maps obtained in vivo and in vitro supports this analysis. In JEG3 cells, after in vivo labeling, spot 1 is the major labeled peptide. In contrast, when Fos is phosphorylated in vitro by PKA, spot 3 is the major labeled peptide. These observations fit with spot 3 resulting from a single phosphorylation event by PKA, whereas in vivo, a second phosphorylation event would occur, leading to phosphorylation of residue 373/374 and shifting the major labeled peptide from spot 3 to spot 1. From its mobility relative to spot 3, spot 4 is likely to be the C-terminal peptide cleaved between residues 360 and 361 phosphorylated at a serine other than serine 362. Spot 2 would then be this same phosphopeptide cleaved between residues 359 and 360 (Fig. 3A).

TABLE 1. Enhancement of the transforming capacity of Fos by mutation of serine 362[a]

Transforming DNA encoding:	No. of foci		
	Expt 1	Expt 2	Expt 3
c-Fos	55	32	70
FosSerA	229	151	163
FBR-MuSV(v-Fos)	298	158	197

[a] 208F cells were transfected with 6 μg of transforming plasmid DNA encoding v-Fos, c-Fos, or FosSerA. Focus assays were performed as described in Materials and Methods. Foci were counted 3 weeks after transfection.

We have shown that phosphorylation of Fos protein at the C-terminus occurs at serine residues 362 and 373/374, whereas serines 368, 369, and 371 (mutant FosSerB) remain unmodified (Fig. 3C and 5C). However, mutant FosSerB when used in a transrepression assay is unable to downregulate the c-*fos* gene promoter (29), suggesting that Fos transrepression activity requires secondary-structure features which are disrupted by mutation of serines 368, 369, and 371 to alanine. Indeed, repression of the *fos* promoter by Fos protein is not compromised if serine residues 362 to 364 are mutated to aspartic acid, thus producing an overall negative charge (29).

The importance of phosphorylation as a regulator of activity of transcription factors has been amply demonstrated (5). In particular, PKA has been involved in activation of the CRE-binding protein (17), in translocation of NF-κB to the nucleus, probably via phosphorylation of its inhibitor, IκB (39), and in activation of the oncogenic potential of v-ErbA, the mutated form of thyroid hormone receptor (16). It has already been well established that the PKA pathway is involved in *fos* gene induction. Indeed, treatment with cAMP analogs resulted in c-*fos* gene expression in macrophages (7), NIH 3T3 cells (8), PC12 cells (21), and THP-1 cells (2). This induction appears to be mediated by multiple sequence elements in the c-*fos* gene promoter (4, 15, 37). Recent reports suggest that the PKA pathway might be involved at other levels in the control of Fos protein activity. It has been shown that a protein responsible for Fos retention within the cytoplasm of serum-starved cells is inactivated upon treatment of cells with a cAMP analog or agents elevating the intracellular concentration of cAMP (32). Other works have led to the identification of a protein, IP-1, which inhibits the DNA-binding activity on the Fos-Jun complex binding capacity and is modulated by PKA (1). Treatment of IP-1 activity-containing extracts with PKA alleviates their inhibitory activity, suggesting that phosphorylation of IP-1 regulates its interaction with the Fos-Jun complex (1). Our results add yet another level of control of Fos protein activity by PKA by showing a direct involvement of PKA in Fos protein phosphorylation. In the two examples mentioned above, treatment of cells with activators of the PKA pathway led to induction of Fos protein activity either by suppressing an inhibitory effect (1) or by allowing nuclear translocation (32). Since phosphorylation of the C terminus of Fos is required for transrepression by Fos, phosphorylation by PKA acts as a repressor of Fos activity by allowing downregulation of the c-*fos* gene promoter. It is interesting that PKA can be involved, directly and indirectly, in both activating and repressing Fos protein activity. The complexity of this regulatory control is further enhanced by the possible cross-talk between the PKC pathway, activated by phorbol esters, serum and some growth factors, and the PKA pathway (1, 30, 37).

How does direct interaction occur between PKA, a cytosolic protein, and Fos, usually detected in the nucleus of induced cells? Fos could be modified in the cytoplasm immediately after synthesis and prior to nuclear translocation. However, the kinetics of Fos expression and of Fos phosphorylation do not favor such hypothesis. The observation that the PKA catalytic subunit is translocated to the nucleus of bovine epithelial cells shortly after treatment with

forskolin (27) fits with a delayed phosphorylation of Fos compared with its synthesis. Thus, direct phosphorylation of Fos by PKA most likely occurs in the nucleus.

Phosphorylation by PKA of a serine residue located at the C terminus of Fos might be a general feature of the members of the Fos family, since the consensus sequence for PKA is highly conserved among these members (11, 23, 28, 42, 43). We suggest that the unmodified form of the Fos family of proteins forms complexes with Jun family members to activate transcription of genes containing AP-1 site. Because sustained expression of Fos can cause cellular transformation, expression of the Fos protein must be regulated. Modification of the Fos protein by phosphorylation with PKA then allows it to act as a regulator of its own synthesis by downregulating *fos* gene expression at a transcriptional level (29, 36). The v-Fos protein has an altered C terminus due to in-frame deletion and, as a result, is missing the PKA site. Consequently, it cannot suppress transcription of the c-*fos* gene. The role of phosphorylation of Fos in controlling its unregulated expression is therefore critical because mutant FosSerA, unable to repress c-*fos* expression, transforms cells with an efficiency similar to that of v-Fos protein (Table 1). Since the *fos* gene is expressed in response to a wide variety of external signals, its regulation by PKA phosphorylation is a major regulatory mechanism during growth and differentiation of cells.

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